

ICE/CED3-like Proteases as Therapeutic Targets for the Control of Inappropriate Apoptosis

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Excessive or failed apoptosis is a prominent morphological feature of several human diseases. Many of the key biochemical players that contribute to the highly ordered process of apoptotic cell death have recently been identified. These include members of the emerging family of cysteine proteases related to mammalian interleukin-1 β converting enzyme (ICE) and to CED-3, the product of a gene that is necessary for programmed cell death in the nematode *C. elegans*. Among a growing number of potential molecular targets for the control of human diseases where inappropriate apoptosis is prominent, ICE/CED-3-like proteases may be an attractive and tangible point for therapeutic intervention.

Complex multicellular organisms employ numerous mechanisms to protect and maintain the integrity of the cellular components from which they are comprised. In the event of questionable or irreparable cell damage, one seemingly drastic measure available to a compromised cell is to commit suicide and to discretely remove itself for the altruistic benefit of the organism as a whole. Cell suicide is triggered in response to pathogenic invasion (such as by a virus) to halt the spread to neighboring cells, but it also occurs under nonthreatening conditions to replace redundant or unnecessary cells, as occurs in tissue morphogenesis and remodeling, and in the normal turnover of cells that occurs when they have exhausted their overall usefulness within the organism. Cell suicide thus plays an undeniably important role in the development, maintenance and defense of higher organisms, including humans.

The pathological manifestation of cell suicide, known as apoptosis¹, occurs as a result of a highly systematic and deliberate cell death pathway. These types of cell deaths have been appropriately termed 'physiological cell death', which refers to the use of an intrinsic mechanism that exists for the specific purpose of committing suicide, or 'programmed cell death', where in addition to this suicide pathway, death occurs in specific cells at a predetermined time. Apoptotic death is a highly ordered process that is characterized by nuclear changes such as chromatin condensation, fragmentation and margination as well as internucleosomal DNA cleavage (usually resulting in the hallmark DNA laddering), and by ultrastructural changes including cytoskeletal disruption, cell shrinkage and membrane blebbing which then leads to fragmentation of the dying cell into numerous membrane-bound apoptotic bodies that are subsequently engulfed by neighboring cells or professional macrophages in the final resolution of the suicide process. Apoptotic suicide has many advantages over other forms of cell death, owing principally to the membrane integrity that is maintained throughout the entire process. Necrotic cells, for example, leak their constituents into the surrounding extracellular space usually resulting in an inflammatory response.

Inappropriate Apoptosis

The recent explosion in interest in apoptosis is warranted given the substantial evidence that inappropriate apoptosis may contribute to the pathology of several human diseases (Table 1)²⁻⁴. These can be

divided into disorders of excessive apoptosis (such as neurodegenerative diseases or ischemic damage) and those where insufficient apoptosis occurs (such as autoimmune syndromes, cancers and sustained pathogenic infections). In Alzheimer's disease, for example, hippocampal neurons appear to prematurely commit suicide, resulting in progressive and irreversible memory and cognitive losses^{5,6}. Although the provocation that leads to the early suicide of these irreplaceable cells is unknown, it may involve the deposition of the β -amyloid peptide which has been shown to induce apoptosis in neuronal cell cultures^{10,11}. In spinal muscular atrophy, the loss of motor neurons in the spinal cord has been linked to a defect in a gene that encodes neuronal apoptosis inhibitory protein (NAIP) that is homologous to known viral inhibitors of apoptosis (IAPs)^{12,13}. The loss of the protective anti-apoptotic effects of NAIP probably allows motor neuron apoptosis to occur unchecked. The selective neuronal cell death that develops in trinucleotide repeat disorders, such as Huntington's disease, also appears to be apoptotic¹⁴⁻¹⁶, although the link between polyglutamine expansion and progressive cell death is unresolved.

In contrast to the above mentioned disorders, insufficient apoptosis is also associated with several human diseases. For example, many cancers are now believed to be the consequence of failed apoptotic cell death instead of enhanced cell growth as was originally thought. Two gene defects that are highly associated with proliferative disorders (p53 and Bcl-2) are now known to be regulators of the apoptotic process¹⁷⁻²¹. When p53 is dysfunctional, apoptosis fails to occur in part because of failed cell-cycle checkpoint control and the loss of transcriptional activation of death-promoting genes such as *bax*. Overexpression of death-suppressing proteins such as Bcl-2 (which occurs in most follicular lymphomas owing to a t(14;18) chromosomal translocation) also retards the normal suicide process, leading to cell accumulation. Another example of flawed apoptosis is in autoimmune disorders where the failure to remove autoreactive lymphocytes that arise during development, or subsequent to an immune response, occurs²²⁻²⁴. One form of autoimmune disease, lupus erythematosus, may involve a failure to complete apoptosis execution (e.g., at the engulfment stage) since most lupus autoantibodies recognize cryptic epitopes within a distinct subset of polypeptides that are proteolytic cleavage victims in the cell death pathway^{25,26}. Finally, persistent viral infections may be sustained and

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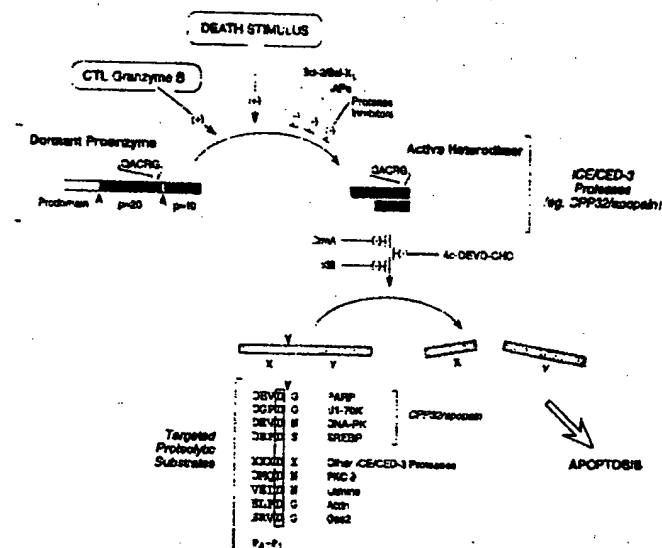


Figure 1. Components of the effector mechanism of apoptotic cell death. Dormant proenzymes of ICE/CED3-like cysteine proteases (such as CPP32/apopain) are proteolytically activated following a suitable death stimulus. The active enzymes cleave a discrete subset of proteolytic targets (following P, aspartic acid residues) which collectively result in the systematic apoptotic death of the cell. Sites for some of the proteins that are known to be cleaved by ICE/CED3-like proteolytic activities are indicated.

propagated because the normal host-cell suicide response that would be engaged following viral infection is cleverly suppressed by anti-apoptotic viral gene products (such as adenovirus E1B 19K protein, the viral IAPs or baculovirus p35)²²⁻²⁷.

A Discrete Biochemical Pathway

Many of the clues which have implicated various components of the cell death pathway have arisen from disease association, genetic analysis and *in vitro* reconstitution of apoptotic events. Collectively, this information has helped define a biochemical pathway that accounts for many of the key events that occur in dying cells *in vivo* (Fig. 1). Many parts of this pathway, particularly those involved in the effector events that mediate the actual cell death process itself, appear to be common to most cell types. At the heart of this process, pro-

Table 1. Some human diseases where inappropriate apoptosis is prominent:

- Neurological
 - Alzheimer's disease
 - Amyotrophic lateral sclerosis
 - Spinal muscular atrophy
 - Neurological stroke damage
 - Parkinson's disease
 - Huntington's disease
- immune System Disorders
 - Autoimmune syndromes
 - AIDS
 - Type 1 Diabetes
- Cardiovascular
 - Ischemic cardiac damage
- Proliferative
 - Solid tumors
 - Follicular lymphoma
- Other
 - Pathogenic (viral) infections
 - Alopecia
 - Aging

teases related to mammalian interleukin-1 β converting enzyme (ICE) and to nematode CED-3 appear to play an essential role. CED-3 was initially identified by genetic analysis of the nematode *C. elegans* as the product of one of two genes (the other being *ced-4*) that were absolutely required for programmed cell death to occur. CED-3 was found to encode a cysteine protease that was highly related to mammalian ICE, an enzyme whose only known function was the proteolytic maturation of pro-interleukin-1 β to the biologically active inflammatory cytokine³⁹⁻⁴². Although this initially implicated ICE itself in mammalian apoptosis, it now seems clear that other ICE homologues are more likely candidates as functional counterparts of CED-3 in higher organisms. This has been supported, for example, by ICE-deficient knock-out mice where no defects in apoptosis appear, with the possible (but controversial) exception of Fas-mediated thymocyte apoptosis⁴³⁻⁴⁶. Several of the substrates that are cleaved by ICE/CED-3 like proteases at the onset of apoptosis have also been identified. These include proteins that function in DNA repair as well as structural proteins and regulatory enzymes⁴⁷⁻⁵⁶. A fundamental principle of apoptotic cell death thus appears to be the proteolytic disabling of key homeostatic- and repair-processes as well as the obvious structural dismantling of the cell that is required to facilitate its breakdown and subsequent packaging into apoptotic bodies. Moreover, ICE/CED-3 like proteolytic activities have been demonstrated to play a role in most if not all of these cleavage events.

Molecular cloning has identified several human homologues of ICE and CED-3 including ICE_h-II (TX, ICH-2), ICE_h-III, ICH-1 (equivalent to murine Nedd2), CPP32 (apopain, Yama), Mch2 and Mch3 (ICE-LAP3)³⁷⁻⁴⁶. Although the precise role and contribution that each ICE/CED-3 family member makes to inflammation or apoptosis is only partly resolved, some clues may exist in their phylogenetic relationships. Members of the ICE/CED-3 protease family cluster into two subfamilies. Those related to ICE (ICE_h-II and ICE_h-III) can be structurally distinguished from those related to CED-3 (ICH-1/Nedd2, CPP32/apopain, Mch2, Mch3). At least some of these proteases, however, have been directly implicated in mammalian apoptosis. CPP32/Apopain (Yama), for example, accounts for the proteolytic activity of 'prICE' (protease resembling ICE) that is responsible for the cleavage of at least three substrates at the onset of apoptosis, including poly(ADP-ribose) polymerase (PARP)^{47,48}, the 70 kDa subunit of the U1 small ribonucleoprotein (U1-70K) and the catalytic subunit of DNA-dependent protein kinase (DNA-PK_α)⁴⁹. CPP32/apopain is also the most highly related of the known human proteases to the *ced-3* 'death-gene' product, including a very high degree of conservation of specific amino acids that are predicted to determine substrate specificity. Selective inhibitors of this enzyme subfamily also inhibit apoptosis in several mammalian systems whereas comparable concentrations of ICE-selective inhibitors are much less effective^{50,51,52-54}. Furthermore, CPP32/apopain is cleaved and activated by CTL-derived granzyme B whereas ICE is not, providing an explanation for how granzyme B launches a suicide response in target cells⁵⁵⁻⁵⁷. Overall, CPP32/apopain appears to play a key role in apoptotic cell death and may be a human counterpart of nematode CED-3, although other human ICE/CED-3 family members undoubtedly make substantial contributions to the suicide pathway. Enzymatic evidence, for example, suggests that Mch3, the most closely related of the known human homologues to CPP32/apopain, has similar catalytic properties as CPP32/apopain and might itself be activated by CPP32/apopain⁴⁵. Murine Nedd2 (ICH-1), on the other hand, was originally identified as a down-regulated gene product in developing mouse brain, suggesting that it might play a role in the substantial apoptosis that occurs in neural tissue preceding maturation⁴⁶. Antisense elimination of Nedd2 results in a delay in apoptotic death following growth factor deprivation but is incapable of preventing death from eventually occurring⁵⁸.

It is not yet entirely clear whether various mammalian ICE/CED-3

family members, particularly those in the CED-3 subfamily, represent enzymes with overlapping biological functions, or whether they are members of a proteolytic cascade with each activated enzyme having responsibilities for the cleavage of a specific subset of targeted substrates. A permutation of both of these possibilities is also plausible. Nevertheless, the essential role that these proteases play in mammalian apoptotic cell death has been well substantiated by protease inhibitor studies. For example, CrmA is a cowpox virus serpin that is a very potent inhibitor of ICE ($K_i \leq 4 \text{ pM}$)²⁵ but can also inhibit the activity of other ICE/CED-3 family members, albeit at substantially higher concentrations (e.g. $K_i \text{ CPP32/apopain} \leq 1 \text{ }\mu\text{M}$)²⁶. When transfected into a variety of mammalian cells, the overexpression of CrmA protects against apoptosis induced by diverse provocations including growth factor withdrawal, Fas and TNF α ²⁷⁻²⁹. Furthermore, when the competitive recognition sequence within CrmA (LVAD³⁰) was modified to favor recognition by CED-3 and CPP32/apopain-like enzymes (DQMD³¹), the modified CrmA also acquired the ability to inhibit nematode programmed cell death³². Another example is the baculovirus p35 protein which inhibits apoptotic cell death in several species and was found to be an equimolar inhibitor of several ICE/CED-3 proteases, including ICE and CED-3 as well as ICE α -II (TX, ICH-2), ICH-1/Nedd2 and CPP32/apopain^{33,34}. Finally, peptide inhibitors of ICE/CED-3 family members have also been shown to prevent apoptosis from occurring^{35,36,37}. More recent studies have demonstrated that inhibitors designed to be selective for CPP32/apopain-like enzymes (e.g., those used on a DEVD tetrapeptide recognition motif) are better inhibitors of apoptosis under identical conditions than those with a selectivity for ICE-like enzymes (e.g., those based on a YVHD recognition motif)^{38,39,40}. Collectively, these studies provide compelling evidence of the critical role that CED-3 plays in nematode apoptosis has been conserved in mammalian cells as well, and that mammalian counterparts of CED-3 play a comparable pivotal role in the cell suicide pathways of higher organisms including humans.

Structure, Catalysis and Inhibition of ICE/CED-3 Proteases

The ICE/CED-3 family of cysteine proteases share many common features both structurally and in terms of their catalytic properties and inhibitor characteristics. This is exemplified by ICE itself and by CPP32/apopain which represent human members of each of the two subfamilies within the larger ICE/CED-3 protease superfamily⁴¹. In addition to having been cloned, the active forms of both enzymes have been purified to homogeneity and their enzymology has been extensively characterized^{42,43}. ICE/CED-3 proteases are all synthesized as inactive proenzymes that are proteolytically processed to their active forms by an unknown mechanism by cleavage at Asp-X junctions. The active mature form of the enzymes are composed of a heterodimer with a large subunit (p20 for ICE, p17 for CPP32/apopain) that contains the catalytic cysteine residue, and a smaller subunit (p10 for ICE, p12 for CPP32/apopain) that contains determinants that govern substrate specificity. The X-ray crystal structure of ICE suggests that two independent functional p20/p10 heterodimers are intimately associated to form a (p20/p10)₂ tetramer with two active sites at opposite ends of the complex^{44,45}.

One distinctive feature of these proteases is the absolute requirement for an aspartic acid residue in the substrate P₁ position, which governs the presence of Asp at all of the proenzyme maturation sites and suggests an autoactivation or inter-enzymatic cascade mechanism for processing to the active forms. The carboxylate side chain of the substrate P₁ Asp is tethered by four residues in ICE (Arg¹⁷⁰, Gln¹⁷¹ from p20 and Arg¹⁰, Ser¹¹ from p10) that are absolutely conserved in all ICE/CED-3 family members. Catalysis involves a typical cysteine protease mechanism involving a catalytic dyad, composed of His²⁷ and Cys²⁸ (contained within an absolutely conserved QACRG pentapeptide) and an 'oxyanion hole' involving Gly²⁹ and Cys³⁰.

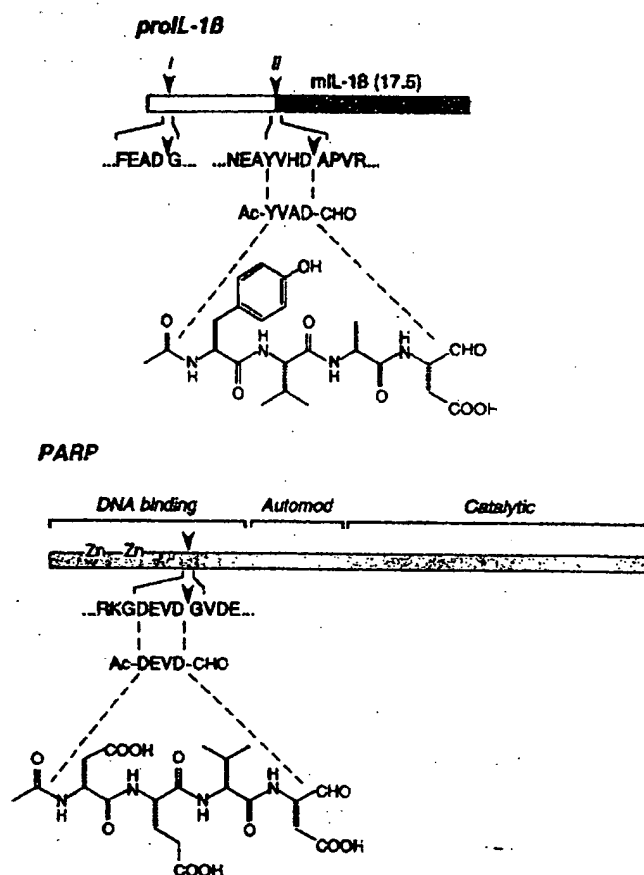


Figure 2. Rational design of tetrapeptide aldehyde inhibitors. The P₁-P₂ tetrapeptide motif within pro-Interleukin-1 β that is recognized by ICE was used as a template for the design of the corresponding acetylated tetrapeptide aldehyde. Using the same strategy, the site within poly(ADP-ribose) polymerase that is recognized by CPP32/apopain was used.

Inhibitors bind, however, in an unexpected non-transition state configuration^{46,47} (which raises important considerations for inhibitor design) with the oxyanion of the thiohemiacetal being stabilized by the active site His²⁷.

A tetrapeptide corresponding to the substrate P₁-P₂ residues is sufficient for specific recognition for both ICE and CPP32/apopain and as a consequence has formed the basis for inhibitor design (Fig. 2)^{48,49}. In addition to the requirement for a P₁ Asp, the P₂ residue in particular appears to be most important for substrate recognition and specificity. ICE, for example, prefers a hydrophobic residue such as Tyr in P₂ (which corresponds to its YVHD cleavage site within proIL-1 β)⁵⁰ whereas CPP32/apopain has a preference for an anionic Asp residue (which corresponds to the DXXD cleavage sites within PARP, U1-70K and DNA-PK α)^{50,51}. Peptide aldehydes, nitriles and ketones are potent reversible inhibitors of these proteases^{48,49,52}, while compounds that form thiomethylketone adducts with the active site cysteine (e.g., peptide (acyloxy)methylketones) are potent irreversible inhibitors⁵³. For example, the tetrapeptide aldehyde Ac-YVAD-CHO (which was designed to mimic the YVHD ICE recognition sequence within proIL-1 β) is a potent inhibitor of ICE ($K_i \leq 1 \text{ nM}$)⁴⁸ but a poor inhibitor of CPP32/apopain ($K_i = 12 \text{ }\mu\text{M}$)⁴⁹. In contrast, the Ac-DEVD-CHO tetrapeptide aldehyde (which was designed to mimic the CPP32/apopain recognition site within PARP) is a very potent inhibitor of CPP32/apopain ($K_i < 1 \text{ nM}$) although it is also a weaker but reasonable inhibitor of ICE, presumably owing to

promiscuity in the S₁ subsite of this enzyme.

Several features plague these peptide-derived inhibitors as a platform for drug design. In addition to their metabolic instability and membrane impermeability, the slow-binding time-dependent inhibition of activity (e.g. $k_{\text{on ICE/AC TWD-CHO}} = 3.8 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$; $k_{\text{on CPD/apoptin/AC-DEVD-CHO}} = 1.3 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) precludes them from the rapid inhibition characteristics that may be necessary to abolish enzymatic activity *in vivo*^{11a}. This may not, however, be an intractable problem since the rapid inhibition properties of CrmA ($k_{\text{on ICE/CrMA}} = 1.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$)²⁵ indicates that in principle these slow binding inhibitor properties can be overcome. Although peptide based inhibitors have served a useful role in defining the enzymology and function of some members of the ICE/CED-3 protease family, they are of limited utility for advanced drug development.

Therapeutic Prospects

Apoptotic cell death is an absolutely essential biological process in complex higher organisms. A key issue, therefore, is whether apoptosis can be discretely and safely modulated for the treatment of human diseases where inappropriate apoptosis plays a major role. Whereas the complete abolition of programmed cell death in nematodes has no adverse consequences, this would certainly not be the case in humans. The selective therapeutic regulation of apoptotic cell death for the purposes of disease modulation therefore presents numerous challenges. On the other hand, exciting opportunities merit the effort to resolve these complexities since viable therapies do not currently exist for many apoptosis-related disorders.

The recent discovery of several key biological components of the apoptotic cell death machinery has exposed some tangible therapeutic targets for both inhibiting and advancing cell death. Amongst these, the ICE/CED-3 proteases are particularly attractive since they are known to play an essential role in mammalian apoptosis and they perform a definable enzymatic reaction against which drugs can be developed. A more complicated issue, however, is how these proteases might be selectively activated for the treatment of disorders where insufficient apoptosis occurs. Although largely undefined, the upstream activation mechanism for these proteases may also be an extremely attractive target for therapeutic modulation. Similarly, other key molecules within the cell death pathway will no doubt emerge as equally attractive targets (e.g., the Bcl-2/Bax family of dimerizing proteins) once their death-modulating activity is better understood.

The rapid development of this field, since the discovery of ICE in 1992, attests to both the importance of these enzymes in human diseases and the fascinating contributions that they make to diverse biological responses such as inflammation and apoptosis. The multiplicity of human ICE/CED-3 isoforms and our evolving understanding of their precise biological role suggests the ideal of a tissue selective molecular target for drug therapy might be an attainable goal.

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